REMARKS

Claims 1-11 were pending. Claims 3 and 9-11 have been canceled without prejudice to renewal or refilling of the original scope. Claim 1 has been amended. No new matter is added. Applicants respectfully request reconsideration of the rejections.

Claim 3 has been rejected under 35 U.S.C. 112, first paragraph. The rejection is made moot in view of the cancellation of the claims.

Claim 6 has been rejected under 35 U.S.C. 112, first paragraph. The Office Action states X-rays are a radiation of higher energy level that may produce damage to cells that may not necessarily be preventable by treating cells with a hyperpolarizing agent.

Applicants respectfully submit that both X-rays and gamma rays are photons. The only difference is the way they are produced (e.g. from decay of a radionuclide vs. the hitting of a target metal with electrons. Radiobiologically they are the same with a RBE of 1.0. Both exist over a wide spectrum of energies. One of skill in the art would reasonably expect to be able to extrapolate from results obtained with one, to the other. Withdrawal of the rejection is requested.

Claims 1-8 have been rejected under 35 U.S.C. 112, second paragraph. The Office Action states that the term "normal" is undefined. Applicants respectfully submit that the term, as used in the present specification, is both defined and is clear to one of skill in the art. As set forth in the specification, "by the term "normal" it is intended to refer to cells that are not transformed, i.e. are not cancer cells. Normal cells are subject to growth control and regulation, for example cells that are normally present in a living organism, primary cell culture, and the like." Claim 1 has been amended to clarify the intended subject matter.

One of skill in the art is familiar with the term "normal cells", and what the term implies in terms of cell biology. Clearly, the usage set forth below illustrates that normal cells are known in the art as non-malignant, or non-transformed cells. As discussed in "Cancer Medicine 6", 2003 BC Decker Inc, Kufe *et al.*:

> Most normal diploid mammalian cells have a limited life expectancy in culture. For example, normal human fibroblast lines may live for 50 to 60 population doublings (the "Hayflick index"), but then viability begins to decrease rapidly unless they transform spontaneously or are transformed by oncogenic agents. However, malignant cells, once they become established in culture, will generally live for an indefinite number of population doublings, provided the right nutrients and growth factors are present. . . . Transformed cells that become established in culture also frequently undergo karyotypic changes, usually marked by an increase in chromosomes (polyploidy), with continual passage. This suggests that cells with increased amounts of certain growth-promoting genes are generated and/or selected during continual passage in culture. . . . Other properties that distinguish transformed cells from their

> nontransformed counterparts are decreased density-dependent inhibition of proliferation and the requirement for growth factors for replication in culture. . . . Normal cells respond to a variety of suboptimal growth conditions by entering a quiescent phase in the cell division cycle.

For the methods of the present invention, the difference in DNA repair between normal and transformed cells is of particular interest. As discussed in "Cancer Medicine 6", 2003 BC Decker Inc, Kufe *et al.*:

> On nuclear DNA damage, normal cells initiate a response that includes cell-cycle checkpoint activation, apoptotic cell death, and transcriptional induction of genes involved in DNA repair. Induction of apoptosis is an important response to DNA damage. Normal cells in G_1 phase prior to the R point will arrest in G_1 phase on sensing DNA damage. This arrest is presumably induced to prevent the replication of damaged DNA. Replication of damaged DNA can result in the incorporation of heritable genetic mutations. If cells are past the R point or within S phase, DNA replication is slowed, again to allow time for DNA repair. If cells sense DNA damage while in G_2 phase, a G_2 cell-cycle arrest will occur. Different types of DNA damage can interfere with normal mitosis, resulting in heritable genetic mutations or cell death.
>
> The tumor-suppressor genes *ATM* and *p53* play an important part in responses to damaged DNA. For example, cells containing mutations in *p53* fail to arrest in G_1 or undergo apoptosis efficiently on irradiation. Cells containing mutations in *ATM* are also deficient for cell-cycle arrest as well as some forms of DNA repair. The p53 protein functions as a transcription factor by binding specific DNA sequences and regulating transcription from promoters containing those sequences. In normal cells, DNA damage induces an increase in p53 levels by inhibiting the normal rapid turnover of the protein.
>
> *ATM* is the gene whose mutation is responsible for ataxia telangiectasia. Immunodeficiency, progressive cerebellar ataxia, radiosensitivity, cell-cycle checkpoint defects, and cancer predisposition characterize this disease. *ATM* encodes a protein containing a phosphatidyl-inositol 3-kinase-like domain, implicating it in signal transduction. Like p53 mutant cells, mutant ATM cells are defective in the G_1/S checkpoint activated after radiation-induced DNA damage. This defect is attributable to the lack of p53 activation that normally occurs, suggesting that ATM may participate in the same pathway as p53. ATM protein, and the related ATR protein, can, in fact, associate with and phosphorylate p53 at its aminoterminal sites. ATM protein, therefore, contributes to the activation and stabilization of p53 by phosphorylating aminoterminal sites during the radiation-induced DNA damage response. ATM protein may play a role in sensing DNA damage and generating the DNA damage signal.

> Although cancer cells use the same cell-cycle machinery as normal cells, the cell-cycle checkpoints in tumor cells are relaxed. Of the scores of proto-oncogenes and tumor-suppressor genes that have been identified to date, most function in signal transduction pathways that mediate mitogenic stimulation. These signal transduction pathways eventually converge on the cell-cycle checkpoint that controls the G_0/G_1 to S phase transition and activate appropriate CDKs. Influencing the transit of this checkpoint has a major influence on the proliferation of normal and tumor cells by affecting both Tc and growth fraction. Increased proliferation, in turn, increases the rate of evolution toward neoplasia. Despite the number and variety of genes involved in signal transduction, relaxation of the G_1/G_0 to S checkpoint controls in tumor cells is mediated, for the most part, by disruption of two pathways, the Rb and p53 growth control pathways. These two genes, individually, are the most frequently mutated in human cancer cells. Disruption of the Rb or p53 pathways probably occurs in virtually every human cancer.

"Normal" cells are not "tumor cells". Although some normal cell lines have been immortalized, they are not tumorigenic - that is - they cannot form tumors *in vivo*. Also, relevant to the present invention, is the fact that many tumor cells overexpress Bcl-2 (unlike most normal cells), which is associated with plasma membrane hyperpolarization. Further, Gilbert *et al* have demonstrated that the Na+K+/ATPase pump was more active in tumor cells overexpressing Bcl-2.

In view of the above amendments and remarks, Applicants respectfully submit that the present claims provide a definite recitation of the subject matter. Withdrawal of the rejection is requested.

Claims 1-2 have been rejected under 35 U.S.C. 102 as anticipated by Fox (1974). Independent Claim 1 has been amended to recite that the hyperpolarizing agent is effective to hyperpolarize the cell membrane and prevent radiation induced cell killing.

Fox teaches that sodium channels of certain neural cells are affected by UV radiation, such that following radiation creates a block in single nodal sodium channels (page 303). The authors conclude from the study that there is a blocking photoreaction that takes place at a specific functional structure of the channel. The location of the photoreaction is at a location where it can be affected by the electric field across the membrane, because the radiation effect was influenced by the membrane potential (page 313). The effects of the radiation described by this paper relate solely to a photooxidation of a membrane protein, and provide no teachings or evidence that there could be an effect of hyperpolarization on viability of the target cells.

Another difference between the present invention and that of Fox is their use of UV irradiation. This is entirely different from ionizing radiation (e.g. photons). UV kills cells by causing characteristic thymidine dimers. The mechanism of cell death induced by ionizing radiation is different and involves different signal transduction pathways. Recent publications demonstrate different mRNA expression profiles following irradiation of cells with UV vs. ionizing radiation, which further substantiates the different mechanisms of cell death induced by UV vs. ionizing radiation.

In view of the above amendments and remarks, Applicants respectfully submit that Claims 1-2 are not anticipated by the cited art. Withdrawal of the rejection is requested.

Claims 1, 2, 4, 5, 7 and 8 have been rejected under 35 U.S.C. 103 as being unpatentable over Gilbert *et al.* (1996). Gilbert *et al.* disclose a method of protecting tumor cells from radiation damage. The Office Action states that one of ordinary skill in the art would have expected that treating normal cells with a hyperpolarizing agent would provide the same degree of protection as treating the tumor cells.

Applicants respectfully submit that one of skill in the art would not have predicted that normal cells would have the same response as tumor cells in response to radiation protection.

As outlined above, the cellular response to radiation damage and killing is complex, typically involving DNA damage and apoptosis. The cellular pathways that control these events include Despite the number and variety of genes involved in signal transduction, relaxation of the G_1/G_0 to S checkpoint controls in tumor cells is mediated, for the most part, by disruption of two pathways, the Rb and p53 growth control pathways. As stated in the above quotes from Cancer Medicine, these are pathways

disrupted in virtually every human cancer, causing a differential in the response of tumor cells and normal cells.

Particularly relevant to the present invention, while most tumor cells express high levels of Bcl-2, the protein is rarely found at significant levels in normals cells. It was shown by Williams *et al.* (2000) (copy attached) that Bcl-2 overexpression results in up-regulation of capacitative Ca2+ entry (CCE). Cells that overexpress Bcl-2 are resistant to SKF-96365-mediated apoptosis and to its inhibition of CCE. Enhanced CCE can be reversed with ouabain, suggesting that Bcl-2-associated plasma membrane hyperpolarization plays a role in up-regulating CCE and may partially explain the antiapoptotic effect of Bcl-2. The demonstration of increased baseline activity of the Na+K/ATPase pump in tumor cells overexpressing Bcl-2 makes it difficult to extrapolate to what one would expect in normal cells, with little Bcl-2 expression and normal pump activity.

Indeed, as evidenced by Neradil *et al.* (2003) (copy attached), in a comparison of the UV protective effects of caffeic acid, there was a pronounced difference between the responses of normal and transformed human skin cells.

One of skill in the art could not have predicted the effect of the present methods on normal cells in the absence of the evidence provided by Applicants.

In view of the above amendments and remarks, Applicants respectfully submit that the present claims are not made obvious by the cited art. Withdrawal of the rejection is requested.

CONCLUSION

Applicants submit that all of the claims are now in condition for allowance, which action is requested. If the Examiner finds that a Telephone Conference would expedite the prosecution of this application, she is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any other fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number STAN-274.

Respectfully submitted,

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UVC-Protective Effect of Caffeic Acid on Normal and Transformed Human Skin Cells *in Vitro*

(UVC / caffeic acid / UV-protective effect / α-tocopherol)

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Abstract. Possible UVC-protective properties of CA, a plant phenolic compound with antioxidant activity, were investigated on human KF1 diploid fibroblast and A431 epidermoid carcinoma cell lines. Cell populations, untreated and treated by antioxidants (CA and α-tocopherol), were irradiated by UVC at the wavelength of 254 nm and their proliferation activity was determined by the MTT assay. The results show a strong protective effect of CA at both concentrations used (55.5 and 166.5 μM): a significant increase of proliferation activity after UVC irradiation was detected in both cell populations growing in the presence of CA in comparison with cells in DMEM only. The described protective effect of CA was more obvious in transformed cells than in normal diploid cells. This protective ability is probably based on the antioxidant and scavenging activities of CA, which seems to be more efficient than α-tocopherol in protection against the cytotoxic effect caused by UVC irradiation.

In a large number of higher plant species, hydroxycinnamic acids are produced as the secondary metabolites of the shikimate pathway from L-phenylalanine or L-tyrosine (Rice-Evans et al., 1996). Caffeic acid (CA), 3,4-dihydroxycinnamic acid, is a biologically active member of hydroxycinnamates. Esters of CA, above all chlorogenic (5-caffeoylquinic) acid, are widely distributed in tissues of many plant species including fruits and vegetables. The highest concentrations of chlorogenic acid are found in coffee, blueberries, apples, and ciders (Cliford, 1999). After dietary intake of CA esters, CA is released by esterases of gut microflora. Free CA is able to pass through the gut wall and was found in human serum and urine (Cremin et al., 2001).

CA has previously been shown to have a multitude of biological activities. A strong antioxidant action of CA was demonstrated on different *in vitro* models. An increased resistance of cells to the oxidative stress that had been caused by t-butyl hydroxyperoxide was proved in the human U937 monocytic cell line. This effect is ascribed to the power of CA, incorporated into cells without any cytotoxic influence, both to reduce glutathione depletion during the oxidative stress and to inhibit lipid peroxidation (Nardini et al., 1998). At micromolar concentrations, CA effectively protects low-density lipoproteins (LDL) from Cu^{2+}-catalyzed oxidation and forms CA:copper complexes responsible for a transient metal chelating activity. This mechanism accounts for the increase of protective activity of CA (Nardini et al., 1995).

Studies on *in vivo* models also led to similar results. Dietary supplementation in rats resulted in a significant increase of α-tocopherol both in plasma and lipoproteins. Without incorporation into lipoproteins, CA may increase their resistance to oxidation (Nardini et al., 1997). CA as the scavenger of ROS also acts in the protection of some proteins from degradation and fragmentation caused by the increase of oxygen radicals. Facino et al. (1995) demonstrated that CA effectively inhibited collagen (type-III) fragmentation induced by superoxide anions and hydroxyl radicals.

CA is also effective as a selective (non-competitive) inhibitor of 5-lipoxygenase, which catalyzes biosynthesis of leukotrienes from arachidonic acid. Leukotrienes are involved in immunoregulation as well as in a variety of diseases including inflammation, asthma and other allergic conditions. For example, CA in concentration 10^{-4} M completely inhibited leukotriene C4 and D4 biosynthesis in mouse mast tumor cells (Koshihara et al., 1984). The xanthine/xanthine oxidase system represents another pathway strongly inhibited by CA; in this system superoxide anion and hydrogen peroxide are the main reactive agents (Facino et al., 1995). Moreover, changes in the xanthine/xanthine oxidase activity may be associated with certain diseases (gout,

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Abbreviations: CA – caffeic acid, CAPE – caffeic acid phenethylester, CPDs – cyclobutane pyrimidine dimers, 1,5-DCQA – 1,5-dicaffeoylquinic acid, DMEM – Dulbecco's modified Eagle's medium, DMSO – dimethyl sulphoxide, LDL – low-density lipoprotein, MTT – methylthiazolyldiphenyl-tetrazolium bromide, (6-4)PPs – 6-4 photoproducts, ROS – reactive oxygen species, UVA – 400-315 nm, UVB – 315-280 nm, UVC – 280-100 nm.

hepatitis) or with the increased incidence of tumours (Chan et al., 1995).

CA esters also exhibit antioxidant properties. Caffeoylquinic acids, i.e. 5-caffeoylquinic acid (chlorogenic acid), 1,5-dicaffeoylquinic acid (1,5-DCQA), and 1,3-dicaffeoylquinic acid (cynarine), are detected in many plant species, particularly in artichoke (Slanina et al., 1999), and in some foodstuffs: coffee, apples, potatoes (Cliford, 1999). Caffeic acid phenethylester (CAPE) isolated from the apian propolis is another biologically active derivative of CA. This compound is cytotoxic for both tumour and virally transformed cells, but not for normal cells (Frenkel et al., 1993). This finding was confirmed by the inhibition of synthesis of DNA, RNA and proteins in consequence of CAPE application in the HeLa cell line (Huang et al., 1996). Similarly as CA, CAPE also inhibits 5-lipoxygenase and manifests the antioxidant activity (Sud'ina et al., 1993).

The protective effects based on the antioxidant activity of CA against UV irradiation, especially UVA and UVB, were demonstrated both *in vitro* and *in vivo* (Saija et al., 1999, 2000). Similar results were also described using other antioxidants for the cell protection against UVA and/or UVB (Kondo et al., 1990; Malorni et al., 1996; Heo et al., 2001). However, there are no data on the possible protective action of CA against UVC. UVC irradiation preferably causes direct damage of DNA due to cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts ((6-4)PPs), and oxidative damage seems to play only a minor role (Kuluncsics et al., 1999; Nishigori, 2000; Yoon et al., 2000).

With regard to the current knowledge on the antioxidant potential of CA and its derivatives after UVA and UVB irradiation, we investigated the possible UVC-protective capacity of CA on two *in vitro* model objects, consisting of normal and transformed human skin cells: KF1 diploid fibroblast and A431 epidermoid carcinoma cell lines. The possible protective effect of CA was assessed by measurement of proliferation activity by means of the MTT assay following UVC irradiation. In comparison with the known antioxidant α-tocopherol, we demonstrated strong UVC-protective properties of CA.

Material and Methods

Cell lines and culture conditions

Human skin fibroblasts (KF1) and human epidermoid carcinoma cells (A431) were chosen for the experiments. The KF1 cell line was diploid, derived in the laboratory using a biopsy sample from a surgically treated donor. The A431 cell line is an established cell line derived from carcinoma epidermis (this cell line was obtained as a gift from the Institute of Molecular Genetics, Prague, Czech Republic). The cells were maintained in DMEM medium supplemented with 10% foetal calf serum, 2 mM glutamine, 100 IU/ml of penicillin and 100 mg/ml of streptomycin at 37°C in an atmosphere of 95% air : 5% CO_2. All the chemicals used were purchased from PAA Laboratories GmbH (Linz, Austria). The cells were subcultivated twice a week.

Antioxidants

Caffeic acid, CA (Fluka, Buchs, Switzerland) was diluted in DMEM at final concentrations 55.5 and 166.5 μM. For comparison with the effect of a known antioxidant, (±)α-tocopherol (Sigma, St. Louis, MO) at concentrations 100 and 300 μM was used in the experiments. (±)α-tocopherol was dissolved in DMSO (Sigma) and the final concentration of DMSO in DMEM was always less than 0.4% v/v, i.e. non-toxic. The final concentrations of CA and (±)α-tocopherol were chosen on the base of previously published data (Kondo et al., 1990; Facino et al., 1995; Nardini et al., 1995; Malorni et al., 1996). Control cell populations were cultivated in DMEM or in DMEM with 0.4% DMSO, respectively.

UV source and irradiation procedure

A TUV 30 lamp (Philips, Eindhoven, Netherlands) was used as a source of UV radiation. This lamp emits UVC radiation at the wavelength of 254 nm at the dose rate of approximately 920 mW/m^2. The cells growing in 96-well microtitre plates were irradiated in a sterile hood. The uncovered microtitre plates were placed at 50 cm below the center of the lamp tube and were irradiated for 30 min. Immediately after irradiation, the culture medium was removed and replaced with fresh DMEM with/without antioxidants in both irradiated and control microtitre plates.

Measurement of proliferation activity (MTT assay)

The proliferation activity of cell populations – untreated (control) and treated by antioxidants – after UV irradiation was determined by the MTT assay based on the detection of mitochondrial dehydrogenase activity in living cells. For each experiment, cells in suspensions at the concentration of 10^4 cells/ml were seeded into 96-well microtitre plates (Nunc A/S, Roskilde, Denmark) in a volume of 200 μl per well. Antioxidants at given concentrations were added to the cell suspensions before seeding into microtitre plates. The cells were allowed to grow under standard conditions for 24 h and then were irradiated as described above. The MTT assay was performed immediately after irradiation and then after reincubation under standard conditions for the following periods: 24, 48, and 72 h. In order to perform the MTT assay, the culture medium was removed; cells were washed with PBS, and 220 μl of DMEM containing MTT (Sigma) at the final concentration 455 μg of MTT per ml of DMEM were

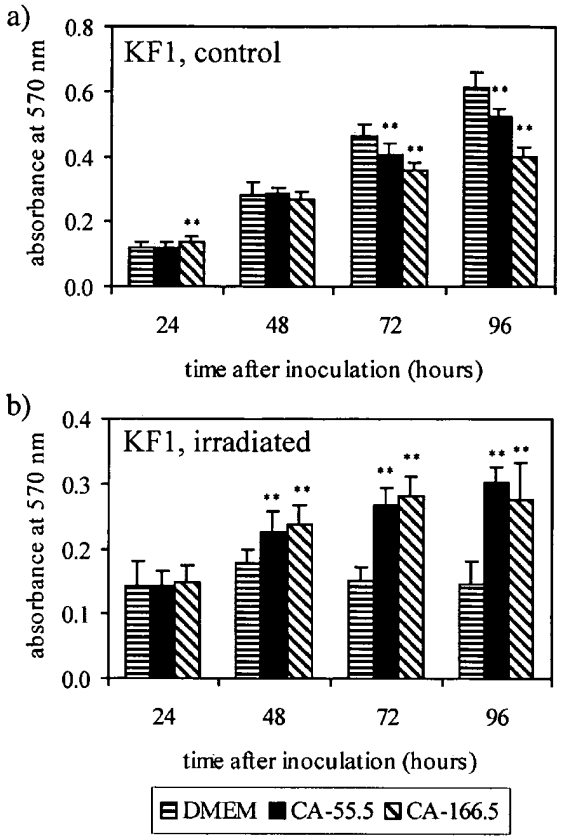

Fig. 1. Proliferating activity (measured using the MTT assay) of control (a) and irradiated (b) KF1 cells growing in DMEM only and in DMEM with CA at concentrations 55.5 μM (CA-55.5) or 166.5 μM (CA-166.5). Mean values ± SD. Statistical significance of difference (Wilcoxon unpaired test): * = $P < 0.05$; ** = $P < 0.01$ as compared with the cell population without CA.

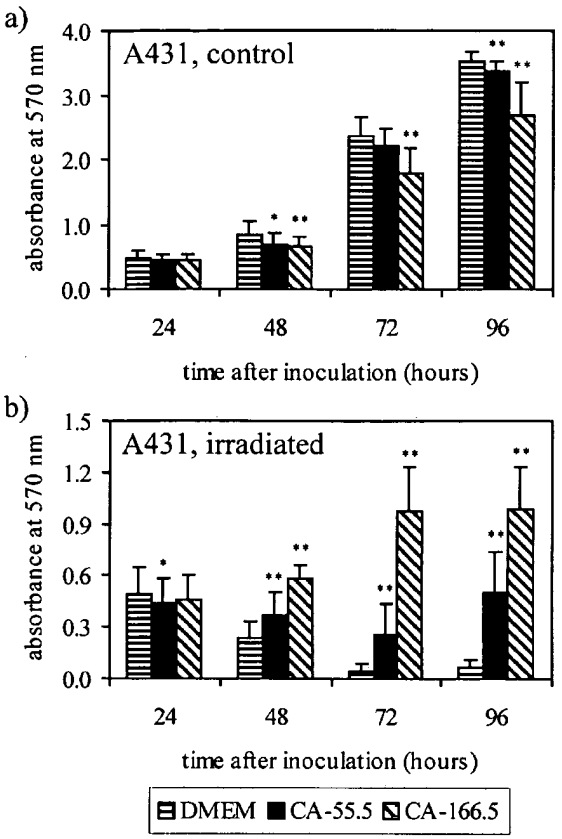

Fig. 2. Proliferating activity (measured using the MTT assay) of control (a) and irradiated (b) A431 cells growing in DMEM only and in DMEM with CA at concentrations 55.5 μM (CA-55.5) or 166.5 μM (CA-166.5). Mean values ± SD. Statistical significance of difference (Wilcoxon unpaired test): * = $P < 0.05$; ** = $P < 0.01$ as compared with the cell population without CA.

added into each well. After 4-hour incubation under standard conditions the medium with MTT was removed and 200 μl of DMSO were added into each well. Absorbance was measured at 570 nm using a Spectra Shell (SLT Laborinstrument GmbH, Salzburg, Austria) microplate reader. At each interval we measured both the irradiated microtitre plates and the control ones, i.e. the non-irradiated ones with the same arrangement of samples.

Statistical analysis

Data were expressed as mean ± S.D. for at least three independent experiments. The significance of differences between mean values was assessed by Wilcoxon unpaired test. The probability of $P < 0.05$ was considered statistically significant. All analyses were carried out using STATISTICA 6 (StatSoft, Inc., Tulsa, OK) and MS-Excel 2000 (Microsoft Corporation).

Results

Our results showed a marked influence of CA on cell proliferation. While in the control non-irradiated cell populations the cell proliferation was significantly reduced by both concentrations of CA used, the same concentrations of CA had a strong protective effect on the cell populations after UVC irradiation.

For the correct interpretation of the potential UVC-protective effect of CA on irradiated cells, we also studied changes in cell proliferation during the cultivation in DMEM containing CA in two different concentrations. Our results proved that the cultivation of cells in DMEM with CA leads to a decrease of proliferating activity (Figs. 1a, 2a) in comparison with cells cultivated in DMEM only, especially after extended reincubation periods, i.e. 48 and 72 h. This effect was similar in both normal (Fig. 1a) and transformed cells (Fig. 2a).

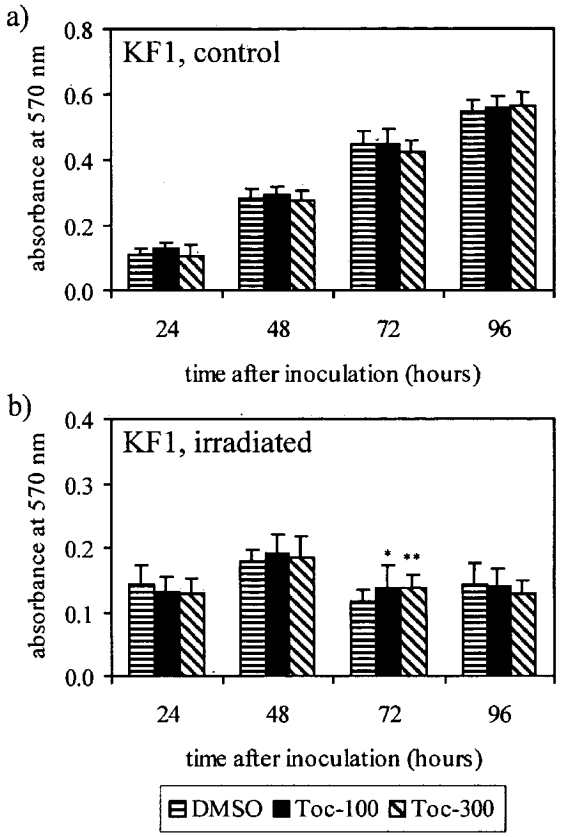

Fig. 3. Proliferating activity (measured using the MTT assay) of control (a) and irradiated (b) KF1 cells growing in DMEM supplemented with 0.4% DMSO and in DMEM with α-tocopherol at concentrations 100 μM (Toc-100) or 300 μM (Toc-300). Mean values ± SD. Statistical significance of difference (Wilcoxon unpaired test): * = $P < 0.05$; ** = $P < 0.01$ as compared with the cell population without α-tocopherol.

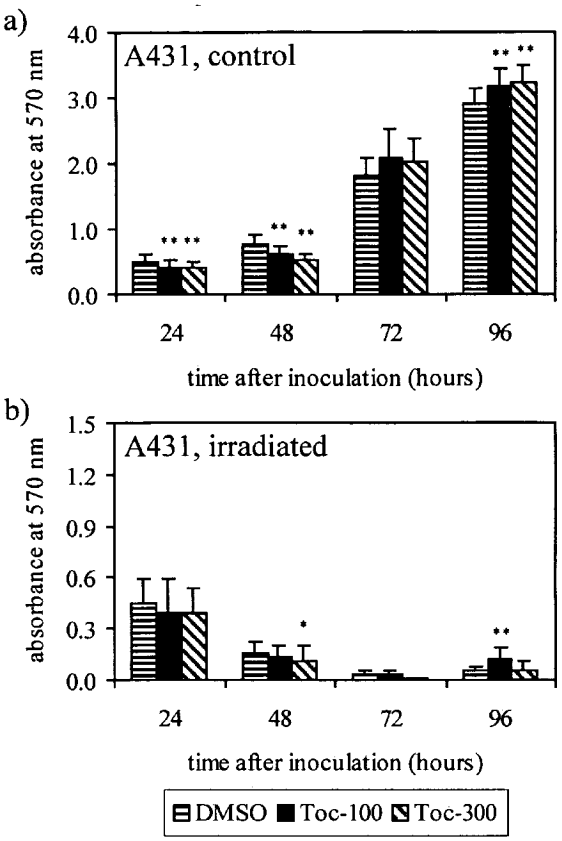

Fig. 4. Proliferating activity (measured using the MTT assay) of control (a) and irradiated (b) A431 cells growing in DMEM supplemented with 0.4% DMSO and in DMEM with α-tocopherol at concentrations 100 μM (Toc-100) or 300 μM (Toc-300). Mean values ± SD. Statistical significance of difference (Wilcoxon unpaired test): * = $P < 0.05$; ** = $P < 0.01$ as compared with the cell population without α-tocopherol.

However, the cultivation of KF1 normal skin fibroblasts in DMEM containing α-tocopherol did not significantly affect their proliferation activity (Fig. 3a). The influence of α-tocopherol on non-irradiated A431 cells was partly different: it reduced the proliferation activity during the first 48 h of cultivation, but the cell proliferation was increased after 96 h of incubation in the presence of α-tocopherol (Fig. 4a).

The UVC-protective effect of CA in both concentrations used (55.5 and 166.5 μM) was clearly demonstrated on normal as well as on transformed cells. A significant difference in the proliferation activity after UVC irradiation was detected in both cell populations growing in DMEM containing CA (Figs. 1b, 2b). This protective effect of CA was more obvious in transformed cells (Fig. 2b) than in normal cells (Fig. 1b). Nevertheless, the dissimilarity between the cytotoxic effect of UVC on CA-untreated cell populations and the stimulation of cell proliferation in CA-treated cells after UVC irradiation was distinct and significant. On the other hand, α-tocopherol in concentrations used in our experiments exhibited no or a very low protective effect on cell populations irradiated by the same dose of UV (Figs. 3b and 4b).

Discussion

Demonstration of the protective effect of CA on UVC-irradiated cell populations contributes new information to the current knowledge on antioxidative and protective properties of the phenolic compounds of plant origin.

UV radiation (waveband C) used in our experiments is preferentially absorbed by DNA. Many investigations employing UVC of the 254-nm wavelength have proved that a vast majority of DNA damage is represented by CPDs and (6-4)PPs. Oxidative damage to purines, pyrimidines, and abasic sites was also detected,

although it seems to play a minor role (Kuluncsics et al., 1999; Nishigori, 2000; Yoon et al., 2000).

Generally, the antimutagenic potential of CA was described in a few studies only. In two short-term genotoxicity assays (Ames assay and Drosophila wing spot test), mutations were induced by aflatoxin B_1; CA was effective in reducing these mutational events whereas α-tocopherol did not show any antimutagenic action (Karekar et al., 2000). Similarly, the potential of CA to reduce acridine orange- and ofloxacin-induced genotoxicity was also evaluated by the Ames assay (Belicova et al., 2001). Nevertheless, both studies conclude that the capacity of CA to protect DNA results from the blockage of mutagenic action by means of CA interacting with a genotoxic compound (acridine orange), from the arrest of metabolic activation of a promutagen (aflatoxin B_1) or from scavenging ROS produced by a mutagen (ofloxacin), respectively (Karekar et al., 2000; Belicova et al., 2001). In view of these facts we cannot assume that the primary UVC-protective effect of CA is due to its interaction with damaged or undamaged DNA molecules.

The MTT assay used for the evaluation of cell viability in our experiments yields results at the level of cell populations. We can suppose that the quantity of direct DNA damage, i.e. the number of CPDs and (6-4)PPs induced by the applied dose of UVC is the same in all experimental variants (control, CA-treated, α-tocopherol-treated) for each cell line and that this type of DNA damage is not affected either by CA or by α-tocopherol.

The antioxidant properties of CA were described in many studies as well as its ability to reduce the oxidative damage of DNA (Wells et al., 1997; Li et al., 2000; Yonezawa et al., 2001; Szeto and Benzie, 2002). The photoprotective activity of CA against UVB was reported both *in vitro* and *in vivo;* the former concerned the evaluation of UV radiation-induced peroxidation in phosphatidylcholine liposomal membranes and the scavenging of nitric oxide (Saija et al., 1999), the latter referred to the CA ability to reduce UVB-induced skin erythema in healthy human volunteers (Saija et al., 2000). In view of these facts, the changes in cell viability and proliferation observed in our experiments represent practically the result of antioxidant action of CA via the reduction of indirect DNA damage caused by ROS.

Differences in the proliferation of CA- and α-tocopherol-treated cell populations may be caused by the higher antioxidant potential of CA in comparison with α-tocopherol. This fact is also supported by the results from different antioxidant assays *in vitro* (Maruta et al., 1995; Chen and Ho, 1997). A similar effect was also reported on human lymphocytes using a comet assay for the evaluation of DNA damage (Szeto and Benzie, 2002).

The sole UVC-protective effect of a plant extract was demonstrated in NIH/3T3 mouse fibroblasts using the comet assay. Ku-35 was the ethanol extract of *Prunus persica* flowers and contained also polyphenolic compounds. The possible action mechanism of Ku-35 against UVC- and UVB-induced DNA damage was ascribed to its antioxidant activity and the efficiency of Ku-35 was higher in comparison with α-tocopherol used as a reference compound (Heo et al., 2001).

However, the increased protection of CA-treated cell populations against UVC may not only be due to the antioxidant action of CA that reduces oxidative DNA damage, but also due to the protection of irradiated cells against oxidative damage of lipid-rich membranes. The strong inhibition of lipid peroxidation by caffeic acid was shown with prevention of human LDL oxidation (Nardini et al., 1995). This UV-protective effect of CA was also described on both cellular (Vieira et al., 1998) and cell-free systems (Laranjinha et al., 1994; Laranjinha and Cadenas, 1999; Hung and Yen, 2002).

In conclusion, our data indicate that CA in the concentrations used can protect cell populations against the cytotoxic effect caused by UVC irradiation. This protective effect is probably based on the antioxidant and scavenger activities of CA, which can reduce the oxidative damage both of DNA and lipid-rich membranes. Despite the fact that the primary damage of DNA induced by UVC irradiation, i.e. CPD and (6-4)PP formation, cannot be reduced by CA treatment, the marked protective effect of CA via its antioxidant properties indicates an important role of plant polyphenolic compounds in the protection of skin cells against UV radiation. Further studies aimed at the evaluation of other different parameters of cellular damage after UVC irradiation will be planned to explore the main possible mechanism of the protective action of CA treatment.

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Advances in Brief

Bcl-2 Overexpression Results in Enhanced Capacitative Calcium Entry and Resistance to SKF-96395-induced Apoptosis[1]

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Abstract

Although there is evidence that changes in cellular ionic concentrations are important early events in apoptosis, the regulation of ion fluxes across the plasma membrane during this process is poorly understood. We report here that Bcl-2 overexpression results in up-regulation of capacitative Ca^{2+} entry (CCE) and that SKF-96365, an inhibitor of CCE, is a potent inducer of apoptosis. Cells that overexpress Bcl-2 are resistant to SKF-96365-mediated apoptosis and to its inhibition of CCE. Enhanced CCE can be reversed with ouabain, suggesting that Bcl-2-associated plasma membrane hyperpolarization plays a role in up-regulating CCE and may partially explain the antiapoptotic effect of Bcl-2.

Introduction

Bcl-2 is one of the most widely studied oncogenes. Despite this fact, its precise mechanism of action has not been clearly elucidated. Overexpression of Bcl-2 is known to convey resistance to apoptosis induced by many different agents, including radiation (1). Results from our laboratory have shown that Bcl-2 has profound effects on plasma membrane functions. Specifically, we have reported that Bcl-2 overexpression is associated with hyperpolarization of the resting plasma membrane potential (2). Furthermore, Bcl-2-overexpressing cells have a higher level of Na^+/K^+-ATPase pump activity (the major contributor to plasma membrane potential) than control cells. Indeed, inhibition of the Na^+/K^+-ATPase pump by ouabain essentially negates Bcl-2-associated radioresistance, suggesting the presence of a relationship between Bcl-2, Na^+/K^+-ATPase pump activity, and radiation-induced apoptosis (3). It was therefore of interest to determine how Bcl-2-associated hyperpolarization affects other plasma membrane functions involved in apoptotic signaling pathways, such as the regulation of the $[Ca^{2+}]_i$.[3]

CCE is the specific gating of Ca^{2+} entry across the plasma membrane in response to depletion of intracellular stores during Ca^{2+} signaling and can be triggered by Tg, an irreversible inhibitor of the ER Ca^{2+}-ATPase (4). CCE is essential for maintaining $[Ca^{2+}]_i$ homeostasis and may therefore be an important regulator of the induction and execution phases of apoptosis, both of which contain Ca^{2+}-dependent components. The results of the experiments described here demonstrate that Bcl-2 overexpression is associated with enhanced CCE, further demonstrating the importance of CCE in the maintenance of cell viability and providing insight into the antiapoptotic effect of Bcl-2.

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[3] The abbreviations used are: $[Ca^{2+}]_i$, intracellular calcium concentration; CCE, capacitative Ca^{2+} entry; Tg, thapsigargin; ER, endoplasmic reticulum.

Materials and Methods

Cell Lines. HL60, a human promyeloid leukemia cell line, was transfected via retroviral gene transfer with the cos MSV-tk-Neo^r-hBcl-2 vector to produce Bcl-2-overexpressing transfectants as described previously (5). PW is a human B-cell lymphoma line that was similarly transfected. The level of Bcl-2 overexpression and associated relative radioresistance has been reported previously (2). Jurkat T-cell leukemia cells were transfected using pREP4 episomal mammalian expression vector and show a 3–5-fold increase in Bcl-2 expression and a 4-fold reduction in apoptosis after 10 Gy of radiation (data not shown).

Measurement of Apoptosis. Flow cytometry was used to determine the sub-G_1-G_0 fraction in fixed cells stained with propidium iodide as described previously (6). Fixed cells were stored at 4°C until they were stained and analyzed on a FACStar flow cytometer (Becton Dickinson, San Jose, CA).

Measurement of $[Ca^{2+}]_i$. Parental cells or Bcl-2-overexpressing HL60, PW, and Jurkat cells were loaded with fura-2/AM (2.5 μM) in HBSS containing 0.5% BSA at 7.0–9.0 × 10^5 cells/ml for 45 min. at 37°C. Cells were washed and resuspended in HBSS/BSA containing 225 nM sulfinpyrazone. Final resuspension buffers also contained SKF-96365 at 10–40 μM or ouabain at 50 μM as indicated. $[Ca^{2+}]_i$ was measured after stimulation with Tg (400 nM) using a Hitachi F2000 fluorescence spectrophotometer with dual excitation of fura-2/AM at 340 and 380 nm and detection of fluorescent emissions at 500 nm. $[Ca^{2+}]_i$ was calculated using the ratio of fluorescence at 340 nm: fluorescence at 380 nm with Ca^{2+} analysis software (Hitachi Instruments, Tokyo, Japan), using the method described by Hitachi. For specific measurement of Ca^{2+} influx, the final wash and resuspension was done in calcium- and magnesium-free HBSS/BSA, and extracellular Ca^{2+} (2 mM $CaCl_2$) was added 45 s after stimulation with Tg. Cells were incubated in Ca^{2+}-free media for 10–30 min before analysis. The resultant rise in $[Ca^{2+}]_i$ was indicative of CCE (7). In prior studies, vector-transfected control cells did not differ significantly from parental cells in any of the measured end points studied.

Results

Enhanced CCE Results from Bcl-2 Overexpression. HL60 and PW control and Bcl-2-transfected cell lines were analyzed to determine the level of CCE after stimulation with Tg. $[Ca^{2+}]_i$ was measured in fura-2/AM-loaded cells in the absence of extracellular Ca^{2+} (Fig. 1*A*). Tg, a well-established inducer of CCE, was added at 20 s to trigger the opening of the plasma membrane calcium release-activated calcium channels. Generally, after the addition of Tg, Ca^{2+} is released immediately from intracellular stores, resulting in an elevation of $[Ca^{2+}]_i$. However, in this experiment, cells were incubated in Ca^{2+}-free media long enough to deplete intracellular Ca^{2+} stores; therefore, no immediate Ca^{2+} release was detected. Only after extracellular Ca^{2+} is replenished 45 s later does $[Ca^{2+}]_i$ rise as the ion crosses the plasma membrane. In both HL60 and PW cell lines, the Bcl-2-overexpressing cells had significantly higher levels of $[Ca^{2+}]_i$ than the control cells (Fig. 1*B*), with a 41% and 60% increase in CCE compared with parental control cells, respectively ($P < 0.005$). Furthermore, Bcl-2-overexpressing cells also had significantly elevated rates of calcium influx compared with similarly treated control cells (Fig. 1*C*). The rate of Ca^{2+} influx in the Bcl-2-transfected cells was 102%

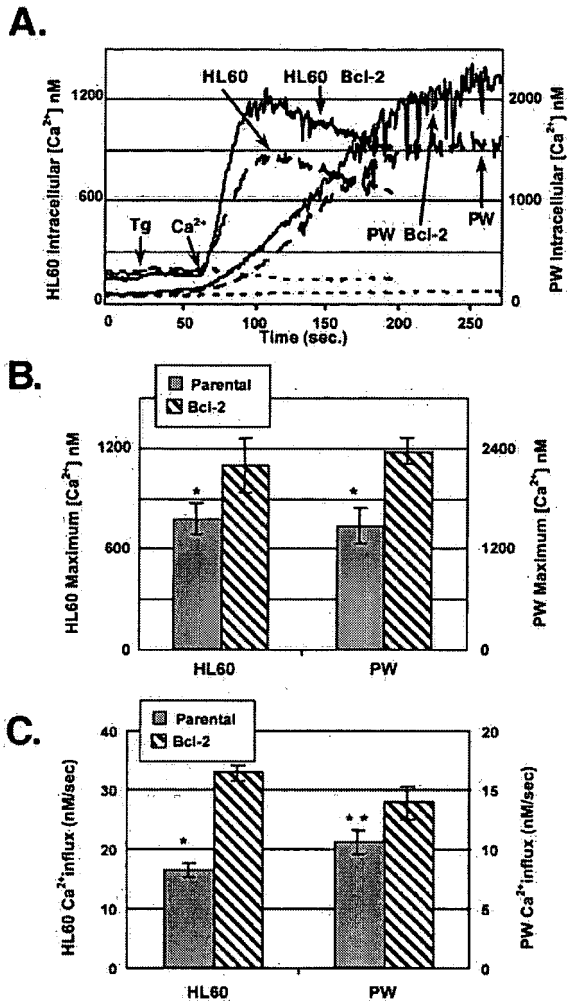

Fig. 1. Bcl-2 overexpression is associated with enhanced CCE in HL60 and PW cells. *A*, representative tracings of $[Ca^{2+}]_i$ induced by Tg stimulation in Ca^{2+}-free media followed by replenishment of extracellular Ca^{2+}. Short-dashed tracings are controls without Tg addition. *B*, measurement of maximum $[Ca^{2+}]_i$ after Tg-induced CCE. *C*, measurement of the slope of calcium influx. ▨, parental cell lines, ▧, Bcl-2 transfectants. *, $P < 0.005$; **, $P < 0.03$. HL60, $n = 6$; PW, $n = 4$. Error bars, mean ± SD.

and 32% greater in HL60 and PW transfectants ($P < 0.005$ and $P < 0.03$), respectively, compared with parental cells.

Bcl-2 Transfectants Are Partially Resistant to the Effects of SKF-96365 on CCE. Tg causes a strong Ca^{2+} signal by releasing ER Ca^{2+} stores, which is followed by compensatory CCE from the extracellular space. In HL60 parental cells, Tg results in the expected elevation of $[Ca^{2+}]_i$, which is suppressed in a dose-dependent fashion by SKF-96365, an inhibitor of CCE (Fig. 2*A*). In contrast, Bcl-2-transfected HL60 cells are resistant to the inhibitory effect of SKF-96365 on CCE (Fig. 2*B*). It is important to note that the rise in $[Ca^{2+}]_i$ seen in this experiment (Fig. 2) results from both CCE and the release of intracellular Ca^{2+} stores. This may explain the somewhat reduced $[Ca^{2+}]_i$ level observed in Bcl-2-overexpressing cells compared with control cells in the absence of SKF-96365, (see "Discussion"). SKF-96365, at concentrations of 10–20 μM, generally resulted in less than 11% inhibition of the Tg-induced Ca^{2+} signal in the Bcl-2 transfectants as compared with up to 54% inhibition in similarly treated HL60 parental cells ($P < 0.0005$; Fig. 2*C*). As can be seen even at 40 μM SKF-96365, Bcl-2-overexpressing cells showed significantly less SKF-96365-mediated inhibition of CCE than control cells ($P < 0.03$).

Inhibition of CCE with SKF-96365 Results in Apoptosis That Is Partially Inhibited by Bcl-2 Overexpression. Next, the effect of blocking CCE on cellular viability was determined. Cells were incubated in increasing concentrations of SKF-96365 for 24 h before quantification of apoptosis (Fig. 3). A subset of similarly treated cells was also irradiated with 10 Gy before determining the apoptotic fraction. At all concentrations of SKF-96365, as in irradiated cells, the Bcl-2-overexpressing cells underwent significantly less ($P < 0.05$) apoptosis than similarly treated HL60 control cells. These results demonstrate the importance of CCE as a determinant of cell viability, the effects of which are modulated by Bcl-2 (Fig. 2). These results suggest that Bcl-2-mediated protection from SKF-96365-induced apoptosis may be due, in part, to the enhancement of CCE in Bcl-2-transfected cells (Fig. 1).

The Enhanced CCE Associated with Bcl-2 Is Reversed with Ouabain. Because ouabain negates the effect of Bcl-2 on plasma membrane hyperpolarization and radioresistance, it was used to determine the effect of plasma membrane depolarization on CCE. In all three Bcl-2-overexpressing cell lines studied, the level of calcium influx in the presence of ouabain was similar to that of parental cells (Fig. 4). Ouabain, at this concentration, was previously shown to depolarize the plasma membrane potential of Bcl-2-overexpressing cells to the level of parental control cells (2). Depolarization of parental cell lines also resulted in partial inhibition of calcium influx.

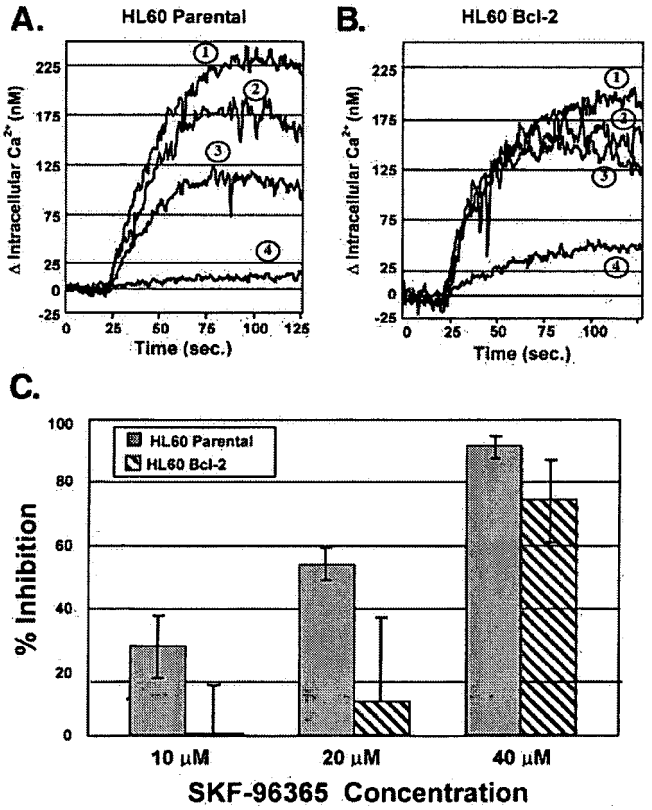

Fig. 2. Bcl-2-overexpressing cells are resistant to SKF-96365-mediated inhibition of the Tg-induced $[Ca^{2+}]_i$ signaling. *A* and *B*, representative tracings showing the effect of increasing concentrations of SKF-96365 on Tg-induced $[Ca^{2+}]_i$ elevations in HL60 parental and Bcl-2-overexpressing cells, respectively. Tracings labeled *1–4* are in the presence of 0, 10, 20, and 40 μM SKF-96365, respectively. Cells were analyzed in Ca^{2+}-containing HBSS; Tg was added at 20 s. Baseline $[Ca^{2+}]_i$ values are normalized to zero. *C*, pooled analysis, expressed as the percentage of inhibition of maximal $[Ca^{2+}]_i$, showing that Bcl-2-overexpressing cells are resistant to the inhibitory effects of SKF-96365. 10 μM SKF-96365, $n = 11$, $P < 0.0005$. 20 μM SKF-96365, $n = 12$, $P < 0.0005$. 40 μM SKF-96365, $n = 6$, $P < 0.03$. *Error bars*, SD.

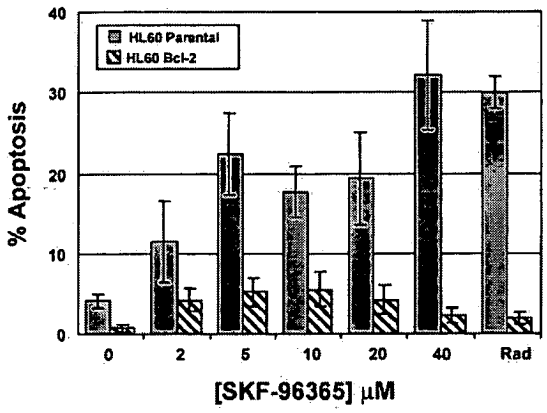

Fig. 3. SKF-96365 induces apoptosis in HL60 cells, and Bcl-2 protects cells from SKF-96365-induced apoptosis. Cells were treated with increasing concentrations of SKF-96365 or with 10 Gy of radiation, as indicated. Apoptosis was measured at 24 h as the fraction of cells containing sub-G_0-G_1 DNA content. $P < 0.05$. *Error bars*, SD.

This observation suggests that up-regulation of CCE is a consequence of Bcl-2-associated plasma membrane hyperpolarization, which decreases the susceptibility of cells to apoptosis.

Discussion

CCE is essential for the maintenance of Ca^{2+} homeostasis in nonexcitable cells (8). Cells use two sources of Ca^{2+} for generating signals: (*a*) Ca^{2+} release from intracellular stores; and (*b*) CCE, which plays a central role in many aspects of cell signaling and is inducible by a wide range of stimuli (4). It is depletion of ER Ca^{2+} stores that initiates CCE to potentiate cytoplasmic Ca^{2+} signals and refill intracellular Ca^{2+} reserves through the action of the ER Ca^{2+}-ATPase pump. Maintenance of intracellular Ca^{2+} stores appears to be crucial to the well-being of cells because prolonged depletion of these stores can induce apoptosis (9). Therefore, cells with efficient mechanisms for replenishing intracellular stores, such as enhanced CCE, may be less likely to undergo apoptosis and may have a significant survival advantage after exposure to apoptotic stimuli.

Recent data also suggest that normal $[Ca^{2+}]_i$ homeostasis plays a fundamental role in both the generation and execution of the apoptotic pathway. For example, Ca^{2+} is required for endonuclease activity that results in DNA fragmentation, and alterations in mitochondrial Ca^{2+} homeostasis result in membrane depolarization, cytochrome *c* release, and caspase activation. Depletion of the ER Ca^{2+} stores appears to be an important apoptotic signal. Interestingly, others have reported that Bcl-2-overexpressing cells retain higher intralumenal Ca^{2+} levels than control cells, making Ca^{2+} store depletion more difficult. Increased retention of ER Ca^{2+} by Bcl-2-overexpressing cells may be due to overexpression of Ca^{2+}-ATPase, the Ca^{2+} pump that sequesters Ca^{2+} in the ER (10). These findings, however, fail to explain the results presented in Fig. 1, in which cells incubated in Ca^{2+}-free media are depleted of intracellular Ca^{2+} stores as indicated by the failure of Tg to elicit an immediate Ca^{2+} release. In the presence of Tg, addition of Ca^{2+} to the media resulted in the rapid accumulation of Ca^{2+} in the cytosol. Because Tg inhibits Ca^{2+}-ATPase, refilling of the ER stores is not possible. Therefore, the rise in $[Ca^{2+}]_i$ observed after the addition of Ca^{2+} to the media is a reflection of CCE across the plasma membrane that is independent of ER Ca^{2+}-ATPase function. Both the magnitude and rate of CCE were greater in cells overexpressing Bcl-2 as compared with parental control cells. This observation is further substantiated by the relative insensitivity of Bcl-2-overexpressing cells to SKF-96365 (Fig. 2).

It is notable that the baseline $[Ca^{2+}]_i$ response to Tg in Fig. 2 is somewhat reduced in Bcl-2 transfectants as compared with similarly treated control cells. This observation is in agreement with the findings of Lam *et al.* (11), who reported that Bcl-2 was associated with reduced efflux of Ca^{2+} from Tg-sensitive ER stores. They suggested that it was possible that inhibition of ER Ca^{2+} store depletion may interfere with the signal to trigger CCE and thereby result in reduced Ca^{2+} influx. This interpretation leads to an apparent discrepancy in comparison to our findings, which can be explained by differences in the experimental technique used. We analyzed cells that had been incubated in Ca^{2+}-free media before the addition of Ca^{2+} at a specified time point. This resulted in a more specific measurement of Ca^{2+} influx and revealed enhanced CCE in Bcl-2-transfected cells (Fig. 1). The results presented here as well as the observation by Lam *et al.* (11) that Bcl-2 transfectants have enhanced uptake of Ca^{2+} into the ER support the hypothesis that the antiapoptotic effect of Bcl-2 is closely associated with alterations in Ca^{2+} homeostasis that protect intracellular Ca^{2+} stores (*i.e.*, enhanced CCE, reduced depletion of ER Ca^{2+} stores, and a greater ability to refill these stores).

The importance of CCE in maintaining cell viability is seen in the

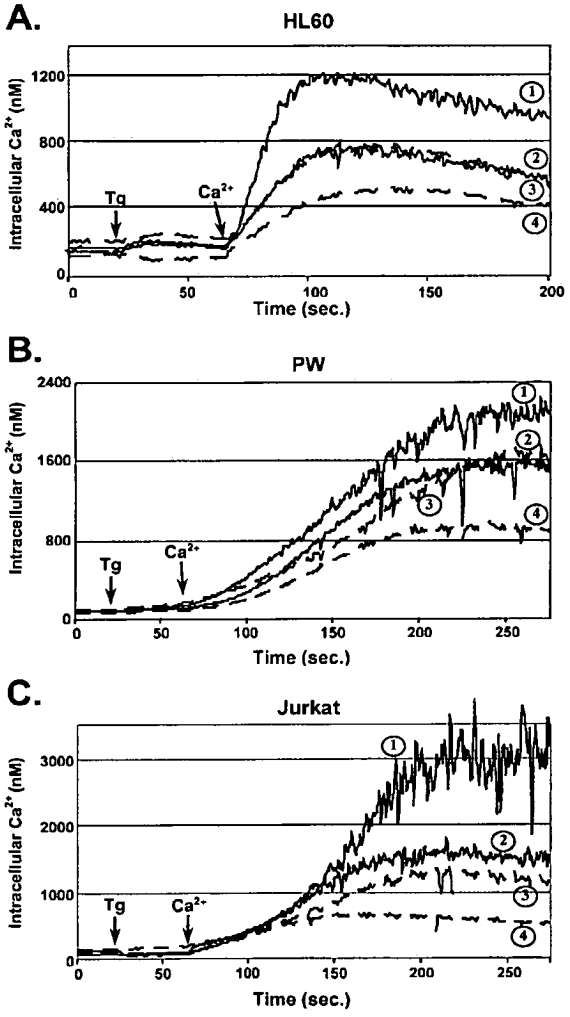

Fig. 4. Depolarization with ouabain reverses Bcl-2-associated enhancement of CCE. Fura-2/AM-loaded cells were analyzed in calcium-free HBSS, Tg was added at 20 s, and extracellular Ca^{2+} was replenished 45 s later. *Dashed tracings* were performed in 50 μM ouabain. *Tracings 1* and *3* represent Bcl-2 transfectants, *tracings 2* and *4* represent parental cell lines. Tracings are representative of eight, six, and two tracings in *A–C*, respectively.

induction of apoptosis by SKF-96365 in HL60 cells (Fig. 3). These results are consistent with those of others who observed that SKF-96365 induced DNA fragmentation in Syrian hamster embryo cells (12). Whereas SKF-96365 has activity on voltage-gated ion channels in excitable cells, it has been widely used and accepted as an inhibitor of CCE in nonexcitable cell types (12–14). Nevertheless, at higher concentrations, SKF-96365 may have other, less well understood effects and should be used with caution (15, 16). The observation that Bcl-2-overexpressing cells are resistant to SKF-96365-induced apoptosis is novel and adds support to the idea that the protective effect of Bcl-2 is related to its modulation of Ca^{2+} homeostasis. This hypothesis is further supported by the observation of He *et al.* (9) that the antiapoptotic effect of Bcl-2 is lost at low concentrations of extracellular Ca^{2+}, further suggesting that an adequate supply of extracellular Ca^{2+} is required for the antiapoptotic function of Bcl-2.

Our findings, as well as those reported by others, suggest that gating of ions across the plasma membrane is critically important to the regulation of apoptosis. Killoran and Walleczek (17) have recently shown that CCE levels are suppressed within minutes after irradiating Jurkat cells and normal human peripheral blood leukocytes with 10 Gy, further emphasizing the importance of CCE in maintaining cell viability. In addition, others have established a fundamental role for K^+ ions in apoptosis (18). Cell shrinkage, which is a very early event in the apoptotic process, can only occur after K^+ efflux. Normal intracellular levels of K^+ inhibit both apoptotic DNA fragmentation and caspase-3 protease activation, suggesting that intracellular K^+ loss must occur early during apoptosis. In addition, there seems to be a tight coupling between cell shrinkage, K^+ efflux, and changes in mitochondrial membrane potential that are independent of DNA degradation and can be largely caspase independent, depending on the particular signal transduction pathway used. Therefore, the control of K^+ gating across the plasma membrane may play an integral role in the initiation of signal transduction pathways involved in apoptosis. We have reported previously that Bcl-2 overexpression is associated with a higher level of Na^+/K^+-ATPase activity, which could block the loss of K^+ from the cell via either (*a*) compensation for K^+ loss by pumping more K^+ back into the cell or (*b*) establishment of a hyperpolarized membrane potential that would inhibit K^+ efflux.

It is known that the influx of Ca^{2+} across the plasma membrane is facilitated by hyperpolarization (19) and inhibited by depolarization (20), suggesting that the plasma membrane potential plays a crucial role in $[Ca^{2+}]_i$ homeostasis. Therefore, plasma membrane hyperpolarization, by providing an electrochemical gradient that favors Ca^{2+} influx, may also help to explain the up-regulation of CCE in Bcl-2-overexpressing cells. Evidence for this is presented in Fig. 4, where plasma membrane depolarization by ouabain, an inhibitor of Na^+/K^+-ATPase, resulted in a marked reduction in the influx of extracellular Ca^{2+} in both parental and Bcl-2-overexpressing cells.

The results reported here show that Bcl-2 overexpression is associated with enhanced CCE, a process that is essential for Ca^{2+} store refilling and cell viability. The previously reported increased Na^+/K^+-ATPase activity and resultant plasma membrane hyperpolarization associated with Bcl-2 may provide a mechanism of action to explain the observed enhancement in CCE. These data further establish the plasma membrane as a regulator of early signaling events in apoptotic pathways, particularly in the control of the intracellular ionic environment. These results suggest that altered membrane potential, Na^+/K^+-ATPase activity, and/or CCE may be important mediators of the enhanced radioresistance of Bcl-2-overexpressing tumor cells and have implications for the development of novel strategies for sensitizing these cells to radiation therapy.

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